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Molecular Phylogeny of the *Dorcus velutinus* Species-group (Coleoptera, Lucanidae) Inferred from the Mitochondrial 16S rRNA Gene Sequences, with the Special Reference to the Taxonomic Status of its Japanese and Taiwanese Members

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Abstract Phylogenetic relationships in the *Dorcus velutinus* species-group, consisting of *D. velutinus*, *D. carinulatus*, *D. taiwanicus* and *D. japonicus*, are examined based on the mitochondrial 16S rRNA gene sequences for re-evaluating the taxonomic status of Japanese *D. japonicus* and Taiwanese *D. carinulatus*. The relatively large sequence divergence in the *D. velutinus* species-group suggests that all the four taxa of this species-group (including *D. japonicus* and *D. carinulatus*) should deserve the status of valid species. The resultant phylogenetic trees indicate that the species-group is divided into two subgroups, one of which consists of *D. velutinus* and *D. taiwanicus*, whereas the other of *D. japonicus* and *D. carinulatus*. This agrees well with their hypothetical affinities based on their morphology.

Introduction

Dorcus velutinus J. THOMASON, 1862 was originally described from India, and later recorded also from Taiwan (MIWA, 1927) and Japan (TAKAI, 1981, misspelled as "*D. veltinus*" in his paper). NAKANE and MAKINO (1985) reexamined Taiwanese and Japanese specimens, and concluded that both the Taiwanese and Japanese forms were specifically distinct from *D. velutinus* from India. Consequently, they described the former as *Dorcus taiwanicus* and the latter as *D. japonicus* (NAKANE & MAKINO, 1985). Besides these, other two species allied to *D. velutinus* have been described from Asia: i.e., *D. ursulus* ARROW, 1938 from Bhutan and *D. carinulatus* NAGEL, 1941 from Taiwan. Of these, as mentioned below, *D. carinulatus* described on the basis of a single male specimen has been taxonomically problematical, because its diagnostic characters mentioned in NAGEL's (1941) description were not so clear, and, what is worse, the unique type specimen deposited in the Hannover Museum was destroyed during the World War II (KRAJCIK, 2003). BENESH (1960) suppressed *D. carinulatus* as a junior synonym of *Metallactulus parvulus*. MAES (1992) followed him, and when establishing a new genus, *Velutinodorcus* MAES, 1992 on the basis of *D. velutinus* as its type

species, he moved three species allied to *D. velutinus* except *D. carinulatus* (i.e. *D. ursulus*, *D. taiwanicus* and *D. japonicus*) to this new genus. On the contrary, MIZUNUMA and NAGAI (1994) considered *D. carinulatus* to be a valid species, and regarded *D. taiwanicus* as a junior synonym of the former. This arrangement was followed by ZHANG (1999) and KRAJCIK (2001, 2003). On the other hand, YANG and CHANG (1997) reported that *D. japonicus* (misspelled as "*D. japonica*" in their paper) was distributed in Taiwan as well as in Japan, and they illustrated its male genitalia. Afterwards, LI (2004) recognized *D. japonicus* and *D. taiwanicus* as two valid species occurring in Taiwan, but he made no comments on the taxonomic status of *D. carinulatus*. Quite recently, NAGAI and FUJII (2005) revised *D. velutinus* and its related species including *D. carinulatus* under consideration. They concluded that *D. carinulatus* should be different from *D. taiwanicus* but identical with Taiwanese *D. japonicus* sensu YANG and CHANG (1997). Further, they regarded *D. japonicus* as a valid species. However, the taxonomic status of *D. japonicus* should be re-evaluated more carefully; according to YANG and CHANG's (1997) illustration, both the external and male-genitalic morphology of *D. carinulatus* (referred to Taiwanese *D. japonicus* by them) were quite similar to those of true Japanese *D. japonicus*. Thus, in the present study, we examine the phylogenetic relationships among the members of the *D. velutinus* species-group (i.e., the members of the genus *Velutinodorcus* sensu MAES' (1992) and *D. carinulatus*) based on the mitochondrial DNA sequences to evaluate the taxonomic status of each species of this group including *D. japonicus*.

Materials and Methods

Insects

We followed the taxonomic treatment of NAGAI and FUJII (2005). Four of the five species in the *Dorcus velutinus* species-group, *D. velutinus*, *D. carinulatus*, *D. taiwanicus* and *D. japonicus*, were examined for DNA analysis. We selected *Dorcus curvidens binodulosus* and *Dorcus titanus pilifer* as outgroups for our analyses. See Table 1 for the detailed collection data of each beetle.

Table 1. List of specimens used in the present study.

Species Name	Sampling Localities	Accession No.
Dorcus japonicus	Isen, Tokuno Is., Kagoshima, Japan	AB 236863
Dorcus carinulatus	Fengyuan, Taichung, Taiwan	AB 236864
Dorcus taiwanicus	Habonshan, Nantou, Taiwan	AB 236865
Dorcus velutinus	Doi Saket, Chiang Mai, Thailand	AB 236866
Dorcus curvidens binodulosus	Yamanashi, Japan	AB178292*
Dorcus titanus pilifer	Sakyô, Kyoto, Japan	AB178293*

* Taken from previous studies (HOSOYA *et al.*, 2001; HOSOYA & ARAYA, in press).

DNA extraction, amplification and sequencing

We used specimens of adult or larva stocked in 99.5% ethanol. DNA was extracted from small amounts of muscle using either the phenol/chloroform method (HOSOYA *et al.*, 2001) or IsoQuickTM Nucleic Acid Extraction Kit (ORCA Research, Inc., USA). A part of the mitochondrial 16S rRNA gene was amplified by polymerase chain reaction (PCR) (SAIKI *et al.*, 1988) using six primers: 16SB, 16SC, 16SD, 16SH, 16SK and 16SL (HOSOYA *et al.*, 2001; HOSOYA & ARAYA, in press). The method of DNA amplification is described in detail elsewhere (HOSOYA *et al.*, 2001). Amplified DNA was purified by electrophoresis in 2% agarose gel, or PCR product pre-sequencing kit (USB, Cleveland, USA). Nucleotide sequences were determined for both strands with a Thermo sequenase cycle sequencing kit (USB, Cleveland, USA) and a LI-COR Model 4200 Automated DNA Sequencer (LI-COR, Lincoln, USA), or SEQ2000 Dye Terminator Cycle Sequencing with Quick Start Kit and SEQ2000 (Beckman Coulter, USA) using 16SB, 16SC, 16SD, 16SH and 16SH2 primers (HOSOYA *et al.*, 2001; HOSOYA & ARAYA, in press).

Phylogenetic analysis

Sequence data was aligned by CLUSTAL W 1.8 (THOMPSON *et al.*, 1994) at DNA Data Bank of Japan (DDBJ), using default gap penalties. The neighbor-joining (NJ) method was applied to infer the relationships among taxa based on pairwise distance matrix from KIMURA's two-parameter model (KIMURA, 1980), using CLUSTAL W. For maximum-parsimony (MP) analysis, branch-and-bound searches were performed using PAUP 4.0b10 (SWOFFORD, 2002). The confidence levels of each branch were estimated using 1000 bootstrap replications (FELSENSTEIN, 1985).

Baysian phylogenetic analyses were conducted with MrBayes 3.0 (HUELSENBECK & RONQUIST, 2001) under the general time reversible (GTR) model with proportion of invariable site and gamma shape parameter (GTR+I+G). Each Markov chain was started from a ramdom tree and run for 5×10^5 generations, sampling the chains every 100th cycle. All sample points prior to reaching stationary (1000 trees) were discarded as burn-in samples. Data remaining after discarding burn-in samples were used to generate a majority rule consensus tree, where percentage of samples recovering any particular clade represented the clade's posterior probability.

Results

Sequence variations

All sequences are deposited in DDBJ nucleotide sequence databases under accession numbers AB236863–AB236866 (Table 1). All sequences in the present study showed a substantial bias for adenine (A) and thymine (T) (average of 74.4%). Several observations have demonstrated the existence of a strong AT bias in the insect mitochondrial genome (SIMON *et al.*, 1994).

The total mitochondrial 16S rRNA gene fragment consists of 996 bp, 176 (17.7%)

Table 2. Pairwise distance matrix from KIMURA's two-parameter model (KIMURA, 1980).

Species Name	1	2	3	4	5
1. *Dorcus japonicus*					
2. *Dorcus carinulatus*	9.4				
3. *Dorcus taiwanicus*	10.2	10.1			
4. *Dorcus velutinus*	10.1	10.0	5.3		
5. *Dorcus curvidens binodulosus*	16.6	18.4	16.6	16.6	
6. *Dorcus titanus pilifer*	23.8	23.4	21.3	21.4	20.7

sites of which were variable. Aligned site of *D. japonicus* consists of 984 bp, *D. carinulatus* of 956 bp, *D. taiwanicus* of 977 bp, *D. velutinus* of 958 bp, *D. curvidens binodulosus* of 981 bp (data from HOSOYA & ARAYA, in press, accession no. AB178292), and *D. titanus pilifer* of 976 bp (data from HOSOYA *et al.*, 2001, no. AB178293), respectively.

The sequence divergence among taxa based on KIMURA's (1980) two-papameter model is summarized in Table 2. The sequence divergence within the *D. velutinus* species-group ranged from 5.3 (*D. velutinus* vs. *D. taiwanicus*) to 10.2% (*D. taiwanicus* vs. *D. japonicus*), and those between the *D. velutinus* species-group and other congeners using as outgroups varied from 16.6 (*D. japonicus* vs. *D. curvidens binodulosus*, *D. taiwanicus* vs. *D. curvidens binodulosus*, and *D. velutinus* vs. *D. curvidens binodulosus*) to 23.8% (*D. japonicus* vs. *D. titanus pilifer*). The sequence divergence between *D. japonicus* and *D. carinulatus* in problem are 9.4%, which is larger than those between *D. velutinus* and *D. taiwanicus* (5.3%).

Phylogenetic relationships

The NJ dendrogram derived from 16S rRNA sequences is shown in Fig. 1. The monophyly of the *D. velutinus* species-group was supported by high bootstrap proportion (BP) value (100%, node 1). Further, the species-group was divided into two clusters, both of which were supported by high BP value: one consisting of *D. velutinus* and *D. taiwanicus* supported by a BP value of 100% (node 2), and the other consisting of *D. japonicus* and *D. carinulatus* with a BP value of 92% (node 3).

As a result of MP analysis, a single most parsimonious tree was obtained (length=431, consistency index=0.85, retention index=0.54, rescaled consistency index=0.46). The resulting topology of the dendrogram of MP tree was identical with the NJ dendrogram, of which three nodes were supported by high BP value≧70% (100% for node 1, 78% for node 2 and 88% for node 3, respectively) (Fig. 1).

The resulting topology of the dendrogram of Bayesian analysis was also identical with the NJ dendrogram, and posterior probabilities (PP) values showed that the three nodes of the Bayesian dendrogram were supported by high PP values≧0.90 (1.00 for node 1, 1.00 for node 2 and 0.93 for node 3, respectively) (Fig. 1).

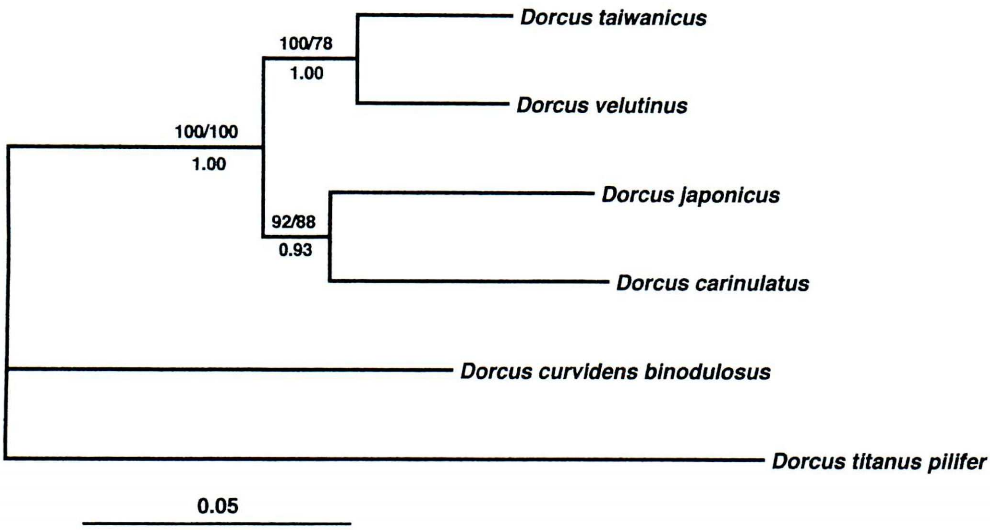

Fig. 1. Neighbor-joining (NJ) dendrogram, with which the topology of those of MP and Bayesian analysis were identical, derived from KIMURA's (1980) two-parameter distances from the mitochondrial 16S rRNA sequences. Bar equals 0.05 substitutions/site. The numbers above internal branches represent the percentage of 1000 bootstrap for nodes supported (left, NJ; right, MP), and those below branches represent the Bayesian posterior probabilities.

Discussion

The sequence divergence in the mitochondrial 16S rRNA gene between *D. japonicus* and *D. carinulatus* (9.4%) observed in the present study is larger than that between *D. velutinus* and *D. taiwanicus* (5.3%), and as much as those between species in the other lucanid genus, *Ceruchus* (10.05%, between Japanese *C. lignarius* and American *C. piceus*) (HOSOYA *et al.*, 2001). These data suggest that the difference in the sequence divergence of mitochondrial 16S rRNA gene between *D. japonicus* and *D. carinulatus* should be considered as inter-specific level, and that the separation between them may have occurred earlier than that between *D. velutinus* and *D. taiwanicus*. These suggestions support the treatment of *D. japonicus* by NAGAI and FUJII (2005) for *D. japonicus*.

As the results of present phylogenetic analyses, the monophyly of the *D. velutinus* species-group was supported by high bootstrap proportions (BP) value (100% in both of NJ and MP) and high posterior probabilities (PP) values (1.00). In addition, it was also revealed that the sequence divergence between each member of the *D. velutinus* species-group and the other *Dorcus* species used as outgroups was relatively large (16.6 to 23.4%). These results may suggest that *Velutinodorcus* MAES is possibly appropriate at least as one of the subgenera of the genus *Dorcus*, although further studies on the phylogenetic relationships among lucanid genera, including *Gnaphaloryx* BURMEISTER, 1847, as well as various species of the genus *Dorcus* are required.

The phylogenetic relationships within the *D. velutinus* species-group inferred in the present study indicate that this species-group is divided into two subgroups, one of which consists of *D. velutinus* and *D. taiwanicus* whereas the other of *D. japonicus* and *D. carinulatus*. This agrees well with their hypothetical affinities based on their morphological similarities (NAGAI & FUJII, 2005). The present results also revealed relatively remote phylogenetic relationship between *D. taiwanicus* and *D. carinulatus*, although both of them are distributed in Taiwan sympatrically (NAGAI & FUJII, 2005). Anyway, further detailed studies on the molecular phylogeny of the *D. velutinus* species-group including another species, *D. urusulus*, are strongly desired in order to discuss their divergence from the zoogeographical point of view.

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要　約

荒谷邦雄・細谷忠嗣：ミトコンドリア16S rRNA遺伝子に基づく*Dorcus velutinus*種群の分子系統と，日本および台湾産の種の分類学的地位に関する検討．—— *Dorcus carinulatus*は，NAGEL (1941)によって台湾中部から得られた，ただ1個体の雄に基づいて記載されたが，タイプ標本が第二次大戦中に消失してしまったこともあって，その正体が不明で，記載以降，分類学的な扱いがたびたび変更されてきた問題の多い種である．永井・藤井(2005)はこの*D. carinulatus* (NAGEL, 1941)を含む*D. velutinus* J. THOMASON, 1862およびその近縁種に関して再検討し，楊・張(1997)が台湾から*D. japonicus* NAKANE et MAKINO, 1985（ヤマトサビクワガタ）として記録した種こそが*D. carinulatus* (NAGEL, 1941)であり，台湾には*D. taiwanicus* NAKANE et MAKINO, 1985と*D. carinulatus*との2種が分布していると結論した．この見解はきわめて妥当であるが，*D. carinulatus*と*D. japonicus*は，外部形態もまた雄交尾器の形態もよく似ており，両者の関係についてはさらなる精査が必要と判断された．そこで本研究では，*D. velutinus*とその近縁種，*D. carinulatus*, *D. taiwanicus*, *D. japonicus*の合計4種に関してミトコンドリア16S rRNA遺伝子に基づく分子系統解析を行い，種群内の系統関係を明らかにするとともに，*D. carinulatus*と*D. japonicus*の分類学的関係についても考察を加えた．解析の結果，*D. carinulatus*と*D. japonicus*の間の塩基置換率は9.4%と，大陸産の*D. velutinus*と台湾産の*D. taiwanicus*の間の値(5.4%)よりも高く，既往のツヤハダクワガタ属の日本産(*Ceruchus lignarius*)と北米産(*C. piceus*)の2種間の値(10.05%)にほぼ匹敵することが判明した．このことから*D. carinulatus*と*D. japonicus*は遺伝的にも別種レベルの違いがあることが裏付けられた．解析の結果得られた系統関係からは，*D. velutinus*種群は*D. velutinus*と*D. taiwanicus*，および*D. carinulatus*と*D. japonicus*からなる2つのサブグループに分れることが判明したが，これは従来の外部形態に基づく本種群内の類縁関係の

推定結果ともよく一致する．また，*D. velutinus* 種群全体の単系統性もきわめて高い確率（NJ と MP, 100%；ベイズの事後確率 1.00）で支持された．Maes (1992) は *D. velutinus* をタイプ種に *Velutinodorcus* 属を創設したが，今回の結果は，この見解にある程度の妥当性があることを示唆したものといえる．今後は，今回の解析では使用できなかった *D. ursulus* Arrow, 1938 はもちろん，*Dorcus* 属のさまざまな種や他属の種も加えた系統解析を行うことで，*D. velutinus* 種群の系統関係を明らかにするとともに，*Velutinodorcus* 属の妥当性や *D. velutinus* 種群の系統生物地理に関しても考察を加えていきたい．

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A Record of *Pyrocoelia atripennis* LEWIS (Coleoptera, Lampyridae) from Is. Okinawa-jima in the Ryukyus

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Through the courtesy of Mr. M. KIMURA and Miss A. OGATA, I was able to examine a specimen of *Pyrocoelia atripennis* LEWIS found in Is. Okinawa-jima. This was collected by an elderly woman living in Okinawa. She said that the species sometimes came into the house during the winter. However, this species is naturally distributed in the Sakishima Islands. In this case, therefore, the firefly seems to have been brought in by someone from the Sakishimas, where it is very common in the winter.

Specimen examined. 1 male, Kochinda, Is. Okinawa-jima, Ryukyus, 16–XI–2003, H. KINJO leg.

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